

**INVESTIGATION OF PRIMARY AND SECONDARY
METABOLITES IN A CHEMICAL STUDY
OF *CORTINARIUS ARMILLATUS*
(*CORTINARIACEAE*, *TELAMONIA*)**

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ABSTRACT. - The fruit-bodies of *Cortinarius armillatus* were investigated for polyols, sugars, phenolic acids, alkaloids and fungal toxins using thin-layer chromatography methods. Arabitol, mannitol, trehalose, fructose, 4-hydroxybenzoic acid, choline and cortinarine A were detected from aqueous and methanolic extracts of the mushroom. The fungal toxins, α -amanitin, orellanine, muscarine, muscimol and bufotenine were not observed. These chemical investigations and acute toxicity studies in mice supported the non-toxicity of *C. armillatus*.

RÉSUMÉ. - Une étude chimique des métabolites primaires et secondaires a été réalisée sur *Cortinarius armillatus* par chromatographie sur couche mince. L'arabitol, le mannitol, le tréhalose, le fructose, l'acide parahydroxybenzoïque, la choline et la cortinarine A ont été mis en évidence dans les extraits méthanoliques et aqueux de *C. armillatus*. Les toxines fongiques, α -amanitine, orellanine, muscarine, muscimol et bufotenine n'ont pas été observées dans ces mêmes extraits. La non-toxicité de *C. armillatus* est confirmée par des études chimiques et pharmacologiques sur la souris.

Key-words: *Cortinarius armillatus*; cortinarine A; arabitol; mannitol; 4-hydroxybenzoic acid; choline.

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INTRODUCTION

Cortinarius armillatus (Fr. : Fr.) Fr. is a large, reddish-brown edible-like mushroom with a clubshaped stalk and irregular reddish bands, single to several, in deciduous woods (Cetto, 1978; Marchand, 1983). To the best of our knowledge, no data exists as to the chemical constituents of *C. armillatus* except pigment composition (Besl *et al.*, 1978). In this work, an examination of polyols, sugars, phenolic acids, alkaloids and other fungal secondary metabolites was undertaken using mono- and two-dimensional thin-layer chromatography (TLC) spraying with selective reagents. On the other hand, acute toxicity studies were also carried out and had never been previously reported.

MATERIAL AND METHODS

1. Material

C. armillatus was collected at Regensburg in Bavaria in October 1993 and preserved by drying after morphological identification from fresh material.

2. Methods

Preparation of extracts

20 g of *C. armillatus* fruit-bodies finely powdered were extracted with methanol (100 ml x 5) by sonication (Ultra Sonik 300 NEY) at room temperature for 1 h.

The methanolic combined extracts were filtered on Durieux filter paper n°113 and evaporated to dryness in a rotary vacuum evaporator at 35-40°C. Aliquots of the residue were added with methanol for thin-layer chromatography analyses (polyols, sugars, alkaloids, fungal toxins) or resuspended in carboxymethyl cellulose for pharmacological investigations.

The powder of mushroom was then re-extracted with water (100 ml x 3) by sonication in the same conditions as previously described. After filtration, the combined aqueous solutions were freeze-dried. The residue was added with water for TLC analyses (polyols, sugars, phenolic acids).

Qualitative determination of polyols and sugars

The methanolic and aqueous extracts were analyzed on silica 60 F254 plates (Merck, ref. 5735) according to Andary *et al.* (1979) in the following solvent systems (V/V): acetone-water (9:1) and methylene chloride-acetic acid-methanol-water (50:5:20:5) in the same dimension. The TLC profiles of the extracts were compared with 0.015 % polyol and 0.1 % sugar standard solutions (W/V) in ethanol-water (98:2, V/V). Rf values were: arabitol, Rf = 0.48; mannitol, Rf = 0.34; fructose, Rf = 0.44; glucose, Rf = 0.40; galactose, Rf = 0.36 and trehalose, Rf = 0.16.

Glucose, fructose and galactose were distinguished using TLC on cellulose plates (Merck, ref. 5577) developed in *n*-butanol-ethanol-water (4:1:2.2, V/V) up to 4 cm solvent front, and sprayed with 0.2% naphthoresorcinol in ethanol (W/V) with 5% sulfuric acid (Rapior *et al.*, 1990). The three hexoses were detected as follows: fructose,

Rf = 0.36, dark fuchsia; glucose, Rf = 0.32, turquoise blue and galactose, Rf = 0.30, pale blue. The polyols and the disaccharide were not revealed by this method.

Qualitative estimation of phenolic acids

A 100 mg aliquot of the water extract was added with water. The solution was adjusted to pH 3 with 10% acetic acid and partitioned with diethyl ether. The organic phase was evaporated to dryness and the residue added with 50% methanol (2 ml). The hydromethanolic solution was analyzed by two-dimensional TLC on cellulose plates (Merck, ref. 5577) developed in 2% acetic aqueous acid (AA) and toluene-acetic acid-water (60:28:1.2, TAW, V/V) (Rapior *et al.*, 1990). The chromatograms were sprayed with 4-nitroaniline reagent (Stahl, 1969). The TLC profile of the extract was compared with 0.1% phenolic acid standard solutions (W/V) in methanol.

Rf values were: 4-hydroxyphenylacetic acid (Rf = 0.81 in AA, Rf = 0.40 in TAW, mauve); 4-hydroxybenzoic acid (Rf = 0.56 in AA, Rf = 0.46 in TAW, pink); 4-hydroxycinnamic acid or p-coumaric acid (Rf = 0.41 in AA, Rf = 0.49 in TAW, blue-grey); 3,4-dihydroxyphenylacetic acid (Rf = 0.75 in AA, Rf = 0.11 in TAW, purple); 3,4-dihydroxybenzoic acid or protocatechuic acid (Rf = 0.63 in AA, Rf = 0.16 in TAW, mauve-grey) and 4-hydroxy 3-methoxybenzoic acid or vanillic acid (Rf = 0.50 in AA, Rf = 0.67 in TAW, purple).

Qualitative detection of alkaloids, quaternary ammonium compounds and α -amanitin

The methanolic extract was chromatographed on cellulose plates (Merck, ref. 5577) using methanol-water-acetic acid (90:5:5, V/V) as mobile phase. TLC plates were sprayed with Dragendorff's reagent modified according to Bregoff-Delwiche (Stahl, 1969) for alkaloids and quaternary nitrogen compounds and, with sulfanilic acid diazotised for α -amanitin, bufotenine and muscimol (Andary *et al.*, 1977).

Concentrations of standard solutions (W/V) and Rf values were: betaine (0.2% in methanol, Rf = 0.56, orange); choline chloride (0.25 % in methanol-water (1:1), Rf = 0.70, brown); muscarine (0.01% in methanol, Rf = 0.82, orange pale); α -amanitin (0.1% in methanol, Rf = 0.65, brownish-pink); bufotenine hydrogenoxalate (0.1% in methanol-water (1:1), Rf = 0.71, pink) and muscimol (0.5% in methanol, Rf = 0.52, pink orange).

Qualitative estimation of other fungal metabolites: cortinarine A and orellanine

The methanolic extract of *C. armillatus* was analyzed for cortinarine A by TLC on silica 60 F254 support (Merck, ref. 5735) in the following monodimensional solvent systems (V/V) : *n*-butanol-acetic acid-water (4:1:1, BAW) and cyclohexane-ethyl acetate (3:1, CHEA) (Caddy *et al.*, 1982) and compared with a methanolic extract of *Cortinarius orellanus* Fr. Cortinarine A appeared as a blue fluorescent spot after exposure to UV light at 254 nm.

Orellanine was detected from the methanolic extract of *C. armillatus* by TLC on cellulose plates (Merck, ref. 5716) in *n*-butanol-hydrochloric acid-chloroform-water (40:20:15:3.8, BCCE, V/V) as mobile phase and compared with a 0.002% orellanine standard solution (W/V) in methanol-water (1:1, V/V) (Rapior *et al.*, 1988). Orellanine

appeared in the form of a dark spot which, after exposure for 1-3 minutes to UV light at 366 nm, produced a bluish-white fluorescence characteristic of orelline, the photodecomposition product of orellanine.

Rf values were: cortinarine A, Rf = 0.86 in BAW and Rf = 0.50 in CHEA; orellanine, Rf = 0.60 in BCCE.

3. Acute toxicity studies

Adult, male and female SWISS mice from 4 to 5 weeks old weighing between 24-26 g and 15-20 g respectively, procured from the Department of Pharmacology (Faculty of Pharmacy, Montpellier) were used for these experiments. The animals were housed 3 male and 5 female per cage (25 x 45 x 15 cm). The room temperature was 22°C and humidity 60 ± 10 %. Artificial light was the only source of light and the animals were set on a 12 hour light/dark cycle. They had free access to commercial pelleted diet (UAR A04) and tap water. The animals had been fasted 18 hours before they were intraperitoneally (i.p.) injected with suspensions of the methanolic extract residue from *C. armillatus* in 3% aqueous carboxymethyl cellulose. The following single doses were given to 6 male (2000, 1000, 500 and 250 mg/kg) and 8 female (2000 mg/kg). All suspensions were prepared in such a manner that 10 ml was given per kg of body weight. The animals were weighed on day 1, 5, 10 and 15, and were sacrificed on day 15 for anatomical observations of thoracic and digestive organs.

RESULTS AND DISCUSSION

1. Polyol and sugar contents

Polyols were generally considered independent of other primary metabolites but they frequently coexist in mushroom extracts with free sugars, which are related carbohydrate compounds. Using the methods by Andary *et al.* (1979) and Rapior *et al.* (1990), arabitol and mannitol were detected from the aqueous and methanolic extracts of *C. armillatus*. Both extracts contained trehalose as also reported for *Cortinarius*, subgenus *Leprocycbe*, section *Orellani* (Rapior *et al.*, 1990) and *Boletus* (Benedict and Tyler, 1968). Fructose was present only in the methanolic extract of *C. armillatus*.

2. Phenolic acid content

Chromatographic examination of the water extract from *C. armillatus* demonstrated only presence of 4-hydroxybenzoic acid. It was observed as the major phenolic acid constituent from seven *Cortinarius* species of section *Orellani* (Rapior *et al.*, 1990).

3. Alkaloid and nitrogen compound contents

C. armillatus contained choline. As reported for *Entoloma* (Maki *et al.*, 1985), presence of choline proved to be of no significance chemotaxinomically. Betaine was not detected by our method.

4. Other fungal metabolite content

Cortinarine A was detected from the methanolic extract of *C. armillatus*. Similar result was obtained on analysis of 59 *Cortinarius* species from the seven subgenera of the *Cortinarius* genus (Tebbett and Caddy, 1983).

On the other hand, the fungal toxins, α -amanitin, bufotenine, muscarine, muscimol and orellanine were not identified from the fruit-bodies of *C. armillatus*. For this reason it is suggested that *C. armillatus* fruit-bodies should now be considered to be potentially non toxic.

5. Toxicity studies

All mice survived the observation period of 15 days. Male and female mice given single i.p. doses of the methanolic extract from *C. armillatus* had no toxic symptoms and no organic pathology up to 2000 mg/kg, two weeks after dosing. This was confirmed by the evolution of the animal weight at different doses for the same period of time. Autopsy of animals sacrificed on day 15, revealed no digestive and pulmonary changes.

CONCLUSION

TLC screening of the methanolic and aqueous extracts of *C. armillatus* revealed the presence of polyols (arabitol, mannitol), sugars (trehalose, fructose), phenolic acids (4-hydroxybenzoic acid), nitrogen compounds (choline) and other fungal metabolites (cortinarine A). Mice given single intraperitoneal doses (from 2000 to 250 mg/kg) of a suspension from the methanolic extract of *C. armillatus* had no symptoms and no organic pathology two weeks after dosing. These chemical studies and pharmacological investigations supported the non-toxicity of *C. armillatus*.

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